REMARKS

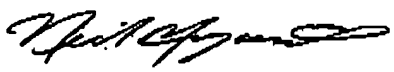
Applicants draw the Examiner's attention to the attached Singh et al. article, which reports mapping of a macular drusen susceptibility locus in rhesus macaques to the homologue of human chromosome 6q14-15. In particular, the Singh et al. article demonstrates that allelic variation in the rhesus macaque *IMPG1* gene, which is the homologue of the human IPM50 gene of the instant invention, shows strong association with retinal drusen formation. Thus, two studies published subsequent to the filing of the instant application, Singh et al. and van Lith-Verhoeven et al., have borne out Applicants' assertion that the currently claimed nucleic acids are useful to analyze ocular diseases and disorders, consistent with the teaching of the specification.

CONCLUSION

In view of the foregoing, Applicants believe that all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,



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Mapping of a macular drusen susceptibility locus in rhesus macaques to the homologue of human chromosome 6q14-15

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Abstract

Rhesus macaques (*Macaca mulatta*) are a natural model for retinal drusen formation. The present study aimed at clarifying whether chromosomal regions homologous to candidate genes for drusen formation and progression in humans are also associated with a drusen phenotype in rhesus macaques. Some 42 genetic markers from seven chromosomal regions implicated in macular degeneration syndromes in humans were tested for whether they identified homologous, polymorphic sequences in rhesus DNA. This was found to be the case for seven markers, all of which were subsequently screened for the presence of potentially disease-predisposing alleles in 52 randomly chosen adult animals from the Cayo Santiago population of rhesus macaques (Caribbean Primate Research Center, PR, USA). The high drusen prevalence expected in the Cayo Santiago colony was confirmed in our sample in that 38 animals were found to have drusen (73%). Logistic regression analysis revealed that some alleles of the rhesus homologue of anonymous human marker *D6S1036* were consistently over-represented among affected animals. Of two candidate genes located in the respective region, allelic variation in one *(IMPG1)* showed strong association with drusen formation. We conclude that one or more genes located at the rhesus homologue of human 6q14-15 are likely to play a role in retinal drusen formation, a finding that represents a first step towards the identification of genetic factors implicated in macular drusen formation in rhesus macaques. This is an important tool for the separation of genetic and environmental factors which must occur before satisfactory management methods can be developed.

Keywords: retina; drusen; ageing macular dystrophy; disease susceptibility; *Macaca mulatta*

1. Introduction

The aetiology and pathogenesis of age-related human macular degeneration (AMD), a major cause for visual impairment in the elderly population, are not well understood. Strong evidence for a genetic contribution to this condition is provided, for example, by the fact that monozygotic twins show strong concordance for AMD, and several genes implicated in early onset forms of macular degeneration have been reported (Gorin et al., 1999; Crabb et al., 2002). In the opinion of several authorities, an initial obligatory symptom and a hallmark of the disease is the formation of drusen, deposits on Bruch's membrane probably derived from the retinal pigment epithelium (Sarks et al., 1999; Hageman et al., 2001; Stone et al., 2001). Association studies such as that on patients from the Beaver Dam Eye Study (Wisconsin, USA) recently identified candidate regions for drusen formation on several human chromosomes, including nos. 5, 6, 12, and 15 (Schick et al., 2003; Seddon et al., 2003). An earlier affected sib-pair analysis yielded evidence for candidate genes on chromosomes 5 and 10 (Weeks et al., 2000). It is likely that, at least in part, these results reflect genetic heterogeneity between and within the populations studied.

It has been known for some time that rhesus macaques (*Macaca mulatta*) represent a natural model for retinal

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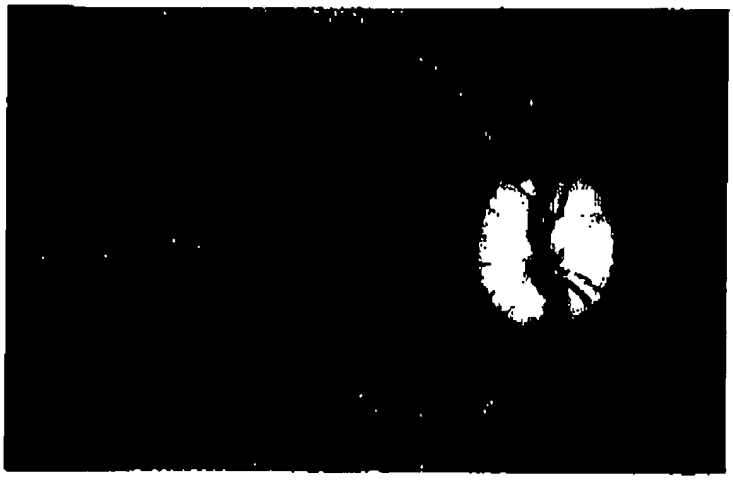

Fig. 1. Fundus photograph of the left eye of a rhesus macaque, showing punctate, coalesced and soft macular drusen with some pigment changes. The photograph is of Cayo Santiago female T69, age 14 years (49 human years).

drusen formation (Stafford, 1974; El-Mofty et al., 1978). Clinically and ultrastructurally, rhesus drusen (Fig. 1) closely resemble human pathology (Ishibashi et al., 1968; Ulshafer et al., 1987; Olin et al., 1995) and, in progressed disease, they may be associated with mildly reduced visual function as measured by electrophysiological means (Engel et al., 1988; Dawson et al., 1989). Drusen formation is usually a relatively rare condition in rhesus macaques, affecting not more than 6% of the aging animals in US primate facilities (Bellhorn et al., 1981; Stafford et al., 1984). Extensive work by one of us (W.W.D.) who investigated this condition in the free-ranging colony of rhesus macaques on Cayo Santiago (CS), Puerto Rico, revealed, however, that almost 60% of the animals living there are affected. Both the prevalence and the severity of drusen on CS increase linearly with age (Hope et al., 1992). As to the causes of the high prevalence, it has been speculated that environmental (diet, light exposure) and genetic factors may both play a role (Hope et al., 1992). A genetic component had to be invoked to explain the significant differences in drusen prevalence observed between different lineages of ancestry on the island.

The CS colony, and all animals derived from that population, are descendants of the 409 animals transferred from India to the Caribbean in 1938 by American zoologist R.A. Carpenter. No new animals have been introduced since then. After 1956, all CS monkeys became individually identified and daily census records were constantly maintained. Animals regularly removed to ensure a constant colony size are housed in large outdoor corrals at the Sebana Seca facility of the Caribbean Primate Research Centre (CPRC) on mainland Puerto Rico. These animals include social group M in which the present work has been performed. Owing to human negligence during World War II, the CS population went through a bottleneck of only 115 animals. Given that the founder population was initially small and became drastically reduced later on, any locus heterogeneity involved in the causation of a particular phenotype is likely to be small on CS, thus rendering the colony particularly suitable for genetic association studies.

We screened a randomly selected subset of CS-derived animals for the presence and severity of retinal drusen formation and assessed the association of the phenotype with allelic variation at markers from chromosomal regions homologous to candidate genes for drusen formation (and possible progression to AMD) in humans.

This is the first study in a subhuman primate aiming at identifying a genetic contribution to macular drusen formation.

2. Methods

2.1. Animals

The study group comprised 52 animals aged 7–22 years. These animals were randomly selected from Cayo Santiago (CS)-derived social group M, maintained at the Sebana Seca field station of the Caribbean Primate Research Centre (CPRC) on mainland Puerto Rico. Since the foundation of the CS colony, the approximately 900 animals residing on the island at any time have only been subjected to behavioural studies. Occasionally, social groups such as group M have, however, been moved to the mainland field station intact to be used for special scientific projects. The study group was randomly subdivided into an initial focus sample of 34 animals (group I), used for exploratory analysis, and a second sample of 18 animals (group II) that served as means of independent verification for any

associations observed in group I. A 2:1 ratio of sample sizes was chosen in order to provide sufficient power for the exploratory stage. Since all positive associations were intended to undergo follow-up by candidate gene analysis, we were able to compensate for the limited sample size by adopting a liberal significance level of 20% for the second stage. Venous blood was obtained from all animals during annual health check-ups, and DNA was extracted from these samples using conventional phenol–chloroform methodology. Eyes were examined under ketamine anaesthesia (10–15 mg kg^{-1}), following pupil dilatation (2% phenylephrine–HCl) using a direct ophthalmoscope, which provides advantages over a hand-held fundus camera in terms of drusen detail and detection (approximately 18 μm), and which is also more convenient to use in the field and in animal quarters. Drusen load was classified as previously described (Dawson et al., 1989). In addition to the subsample of group M, another 13 animals were selected at random from the CS population (Nürnberg et al., 1998) and used for a search of polymorphic markers. In all experiments, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research has been strictly adhered to.

2.2. *Laboratory procedures*

Seven chromosomal regions were chosen for genetic analysis. These segments are homologous to regions harbouring candidate genes for drusen development and macular degeneration in humans, namely *ABCA4* on chromosome 1p22-p21 known to be mutated in autosomal-recessive Stargardt disease (Hoyng et al., 1996; Allikmets et al., 1997); *EFEMP1* on chromosome 2p16 responsible for the causation of Malattia Leventinese (Heon et al., 1996; Stone et al., 1999); a group of loci on 6q14-q21 including *MCDR1* (Small et al., 1993), *PBCRA* (Kelsell et al., 1995), *RP25* (Ruiz et al., 1998), an anonymous locus at 6q14 implicated in autosomal dominant drusen (Stefko et al., 2000), *ELOVL4* mutated in autosomal-dominant Stargardt-like macular dystrophy (Stone et al., 1994; Zhang et al., 2001), *IMPG1* mutated in benign concentric annular macular dystrophy (Felbor et al., 1998; van Lith-Verhoeven et al., 2004), *RIM1* mutated in cone–rod dystrophy (Johnson et al., 2003); loci on 10q25-26 suggested to harbour an AMD-causing gene on the basis of a full genome scan (Weeks et al., 2000); *VMD2* on 11q13 mutated in Best disease (Stone et al., 1992; Forsman et al., 1992; Petrukhin et al., 1998); *TIMP3* on 22q12.1-13.2 known to be associated with Sorsby fundus dystrophy (Capon et al., 1989; Weber et al., 1994; Chong et al., 2000). For a total of 42 polymorphic human markers, located within and around these candidate genes, PCR primers were designed and tested for their capability to yield a product of comparable size in rhesus macaque genomic DNA. The extent of size variation per marker was assessed in a random sample of 13 CS animals (Table 1). All polymorphic markers were first studied for an association with drusen formation in group I, and subsequently analysed in group II.

Table 1
Analysis, in rhesus macaques, of DNA markers from drusen-associated chromosomal regions in humans

Marker	GenBank accession number	PCR product using rhesus DNA	Allele number in rhesus macaques
D1S412	62400	Yes	6
D2S378	75468	Yes	4
D6S430	7289	Yes	7
D6S301	26181	Yes	5
D6S1036	39999	Yes	7
D10S1230	49617	Yes	9
D11S527	89015	Yes	4
D1S424	75820	Yes	1
D1S1170	45340	Yes	1
D1S240	21133	Yes	1
D1S202	22608	Yes	1
D1S2246	81835	Yes	1
D2S2352	64629	Yes	1
D2S2626	36798	Yes	1
D6S1625	46010	Yes	1
D6S460	6530	Yes	1
D6S280	32427	Yes	1
D6S1622	58591	Yes	1
D6S391	10427	Yes	1
D10S1213	35672	Yes	1
D11S4364	44336	Yes	1
D22S273	53198	Yes	1
D22S281	49782	Yes	1
D22S934	150364	Yes	1
D1S236	76703	No	
D1S420	60821	No	
D1S461	76800	No	
D2S2251	6209	No	
D2S2804	73600	No	
D6S249	78799	No	
D6S1671	82504	No	
D6S283	55872	No	
D6S251	18071	No	
D6S252	19859	No	
D6S268	19339	No	
D10S1237	40561	No	
D11S4076	28072	No	
D11S871	146595	No	
D11S4205	45351	No	
D22S275	36928	No	
D22S274	57106	No	
D22S264	85944	No	

All polymorphic markers (i.e. with allele number >1) comprise dinucleotide repeats, except D6S1036 (tetranucleotide repeat) and D10S1230 (tetranucleotide repeat).

PCR assays contained 75 ng of genomic DNA, 50 pmoles of forward and reverse primers, 2.5 U Taq polymerase (Qiagen, Germany) in a total volume of 50 μL 1×PCR buffer (Qiagen, Germany). The standard PCR (Biometra, Germany) protocol comprised denaturation for 420 sec at 96°C, followed by 33–40 cycles with 30 sec annealing at 50–63°C, 30–40 sec elongation at 72°C, and 30 sec denaturation at 94°C. Final elongation was for 600 sec at 72°C.

All markers were run on 6% denaturating PAGE in 1× TBE buffer, and bands were visualized by silver staining. DNA sequencing was done on an ABI Prism 310 genetic analyser or a 3100 Avant genetic analyser, using the DNA sequencing kit-dye terminator cycle sequencing ready reaction (Perkin–Elmer, Foster City, USA).

2.3. Statistical analysis

Logistic regression analysis (Menard, 2001) was used to assess, on a marker-by-marker basis, which alleles potentially predisposed to higher drusen load. To this end, disease severity was first categorised into four classes, depending upon drusen count ('controls', '1–5', '6–10', '>10'). These classes were then taken to represent different ordinal response levels, and the dosage of each allele was modelled as an independent risk factor in group I. Alleles were retrospectively classified as 'risky' if they were assigned a negative factor in the logistic regression analysis. Finally, all risky alleles of a given marker were combined into a single allelic class and the dosage of this risk allele class used as the eventual predictor variable in group II animals. All logistic regression analyses were carried out using the LOGISTIC procedure of the SAS statistics software (SAS Institute Inc., Raleigh, NC).

Haplotype frequencies of markers in one and the same gene were estimated using an implementation ('HAPMAX') of the EM algorithm (Krawczak et al., 1988). Frequency differences between control animals and animals affected by drusen were assessed for statistical significance using a log-likelihood ratio test with a χ^2 approximation (Azzalini, 1996). The level of genetic differentiation between cases and controls was assessed using Weir and Cockerham's F_{ST} (Weir and Cockerham, 1984), calculated using FSTAT Version 2.9.3 (Goudet, 1995).

3. Results

In accordance with earlier observations (Hope et al., 1992), drusen prevalence was found to be high in our study group. There were 14 animals without drusen ('controls'), 24 animals had 1–5 drusen, 7 animals had 6–10 drusen, and 7 animals had more than 10 drusen with occasional signs of coalescence and pigment clumping.

Primers for 24 of 42 human DNA markers (57%) yielded an interpretable DNA product in rhesus DNA, and seven of these (17%) were found to be polymorphic as well (Table 1). The results were therefore largely consistent with previous trans-specific primer hybridisation experiments (Nürnberg et al., 1998). Using genotype data available for 13 unlinked microsatellites and for the homologue of human MHC locus *DQB1*, previously formatted for kinship testing in rhesus macaques (Nürnberg et al., 1998), we assessed the level of genetic differentiation between cases and controls in our sample. Weir and Cockerham's F_{ST} (Weir and Cockerham, 1984) was −0.009, with a 95% bootstrapping confidence interval of [−0.037, 0.010]. There was thus no evidence for a systematic genetic difference between the two phenotypes.

Upon initial identification and clumping of risk alleles in group I ($n=34$), logistic regression analysis revealed that markers *D1S412* and *D6S1036* were potentially associated with drusen formation (post hoc, $p=0.047$ and 0.019, respectively). In group II ($n=18$), however, only *D6S1036* yielded a similar grouping of risk alleles as in group I ($p=0.17$), which was suggestive of a consistent effect and prompted us to undertake a detailed candidate gene analysis of the chromosome 6 region.

Two candidate genes from 6q14–15, namely *ELOVL4* and *IMPG1*, were sequenced in all group I and II animals. In the 3′UTR of *ELOVL4*, a complex allelic variant was detected, del(2183GTT)-2261A→G-del2523C-2597C→T. No association between this polymorphism and drusen load was seen (data not shown). In the *IMPG1* gene, a total of six single nucleotide polymorphisms were identified, namely a G→A exchange in exon 1 (glu22lys), a G→A exchange in exon 7 (ala252thr), an A→T exchange in intron 6 (7 bp upstream exon 7), a silent A→G exchange in exon 10 (position 399), a G→C exchange in exon 13 (ser509thr), and an A→G exchange in exon 10 (met302val). The exon 1 polymorphism occurs in a region that is highly conserved in all mammalian species investigated so far, including humans (GenBank accession number NM001563), chimpanzee (own unpublished result), mouse (BC022970), rat (NM023958), bovine (NM174362), and canine (own unpublished result). In all of these species, lysine is the wild-type allele whereas, in our rhesus macaque sample, the glutamic acid allele was more frequent.

Haplotype frequencies of the six *IMPG1* SNPs were found to differ significantly between affected and non-affected animals ($\chi^2=10.102$, 3 d.f., $p=0.018$). This difference was mainly due to haplotype GGAACA (locus order as specified above) which was found on some 17% of chromosomes of animals with drusen formation, but not in controls (Table 2).

Table 2
SNP haplotype frequencies of the *IMPG1* gene

Haplotype[a]	Affected ($n=38$)	Controls ($n=14$)
GGAGGG	0.449±0.151[b]	0.571±0.285
GGTACA	0.269±0.118	0.321±0.214
AAAAGA	0.064±0.057	0.071±0.101
GGTAGA	0.026±0.036	0.036±0.071
GGAACA	0.167±0.093	0.000
GATGCG	0.013±0.026	0.000
AGTAGA	0.013±0.026	0.000

[a] Alleles are given in the locus order specified in the text.
[b] Maximum likelihood estimate ±2 standard deviations.

4. Discussion

The rhesus monkey appears to provide the best animal model yet reported for human age-related macular drusen. We here present the first investigation of genetic factors possibly associated with macular drusen formation in a non-human primate (*macaca mulatta*), employing a positional candidate approach. Seven genetic markers were found to identify polymorphic sequences in the rhesus genome. One of them, *D6S1036*, was found to be potentially associated with drusen formation. *D6S1036* maps to a ~8 Mb (~8 cM) interval on 6q14-15 containing several genes implicated in human macular degeneration syndromes on functional or positional grounds, namely *RIM1*, *KCNQ5*, *SLC17A5*, *C6orf7*, *MyoVI*, *IMPG1*, *ELOVL4*, *TTK*, *GABRR1* and *GABRR2* (Marcos et al., 2000; Edwards et al., 2001; Zhang et al., 2001; Johnson et al., 2003; van Lith-Verhoeven et al., 2004). Two of these genes, *ELOVL4* and *IMPG1*, were fully sequenced and checked for variation in the respective coding regions of all study animals. Whilst a polymorphism in the *ELOVL4* gene was not found to be associated with drusen, a particular *IMPG1* haplotype (GGAACA) was identified as a strong risk factor for the formation of drusen. Neither the *D6S1036* nor the *IMPG1* risk alleles correlated with age of onset or drusen score.

Our data demonstrate that one or more genes on the rhesus homologue of human 6q are likely to play a role in retinal drusen formation. It is possible that *IMPG1* is directly involved in pathogenesis, but this evidence is as yet inconclusive. Although the genetic factors underlying drusen formation are likely to be homogeneous in the Cayo Santiago population, the fact that not all affected animals carried the *IMPG1* risk haplotype implies that it cannot be the only direct cause of disease in the population. Alternatively, there could be more than a single fundus sign that may be classified as 'drusen' using current clinical methods. Studies are currently underway in order to elucidate the potential role of other candidate genes in the same chromosomal region, including *RIM1* and *KCNQ5*. Further studies are planned either at the functional level (gene expression analyses, immunohistochemistry) or involving the clinical assessment and genotyping of offspring from our current focus group. These studies will expand the understanding of interactions which participate in the pathoaetiology of drusen in rhesus macaques and will serve to validate this species as a primate model for early stage macular degeneration in humans.

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